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Characterization of Enterotoxigenic *Escherichia coli* Isolated from U.S. Troops Deployed to the Middle East

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Enterotoxigenic *Escherichia coli* (ETEC) was a common cause of traveler's diarrhea in U.S. soldiers in the Middle East in 1989 and 1990. To determine which bacterial components would be useful in a vaccine, potential protective antigens (toxin, colonization factor antigen [CFA], and serotype) from 189 ETEC isolates were examined. Nearly half of the isolates expressed both ETEC toxins, 39% had only heat-stable enterotoxin (ST), and 17% had heat-labile enterotoxin (LT). CFA/I was the least common colonization factor antigen (11%), CFA/II was common (34%), as was CFA/IV (31%), and 24% expressed none of these CFAs. Fifty-seven O:H serotypes were found. Serotype O6:H16 was the most common, occurring in 29% of the ETEC isolates, usually with LT-ST and CFA/II. Generally, CFA/II was associated with expression of both toxins, CFA/IV was associated with expression of ST, and none of the CFAs was routinely found with LT. We conclude that ETEC from soldiers in the Middle East expressed a variety of antigens and that an effective vaccine will require multiple protective antigens.

Diarrhea is the most frequent health problem among travelers from industrialized countries when visiting tropical or subtropical areas of the world. Approximately one-third of these travelers develop diarrhea (21). While a number of enteropathogens have been associated with traveler's diarrhea, enterotoxigenic *Escherichia coli* (ETEC) is the most frequent cause and is often isolated from over half of the cases. ETEC isolates are also a primary cause of diarrhea in infants and young children in developing tropical countries (5, 10, 15a).

Diarrhea caused by ETEC is also an important illness for military personnel from industrialized countries when they are deployed to less developed countries. The first reports that associated *E. coli* with traveler's diarrhea were from investigations of outbreaks of diarrhea in British troops stationed in Aden (now part of Yemen) in 1965 (22) and in U.S. troops in Vietnam (4). *E. coli* strains of serotype O148:H28 isolated from individuals at both sites have subsequently been found to be enterotoxigenic. Diarrhea caused by ETEC isolates was a well-documented problem for U.S. forces in Egypt (9, 23, 29), Swedish soldiers in Cyprus (27), and U.S. forces in Saudi Arabia in the recent Persian Gulf conflict (Operation Desert Shield) (11). Investigators in those studies used different techniques to identify enteropathogens, but all document the importance of diarrhea caused by ETEC in deployed troops.

ETEC isolates are identified by their ability to produce heat-labile (LT) and/or heat-stable (ST) enterotoxins (15). In order to colonize the intestine, ETEC isolates express fimbrial antigens on their surfaces called colonization factor antigens (CFAs) (8, 12). The best characterized are CFA/I, CFA/II, and CFA/IV. CFA/I is a rigid, rod-like fimbria, while CFA/II and CFA/IV may contain a mixture of rigid fimbriae and nonfimbrial antigens. ETEC isolates bearing CFA/II always express fibrillar antigen CS3 and may also express fimbrial antigen CS1 or CS2. ETEC isolates bearing CFA/IV always express antigen CS6 and may also express fimbrial antigen CS4 or CS5. Many O:H serotypes are associated with ETEC (15). Reports of ETEC in Asia, South America, and Africa yielded over 50 O:H serotypes (1, 2, 17–19, 24, 31).

No vaccine for use against ETEC diarrhea has been licensed or fully developed for human use. However, an oral cholera vaccine composed of killed whole-cell *Vibrio cholerae* plus the B subunit of cholera toxin provided some protection against LT- and LT-ST-producing strains of ETEC (3, 21). The basis for protective efficacy lies in the fact that LT is nearly identical to cholera toxin (15). Although anti-LT immunity is important in prevention of disease, many ETEC isolates produce ST without LT (5, 15a). ST is poorly antigenic and is unrelated to LT, so other common antigens must be evaluated as potential vaccine components. Both CFAs and O:H antigens have been shown to be protective antigens (7, 14). To determine which bacterial components would be useful in a vaccine against ETEC, we characterized potential protective antigens (toxins, CFAs, and O:H serotypes) of ETEC isolated from U.S. soldiers with diarrhea while deployed to Egypt and Saudi Arabia. ETEC isolates were obtained from earlier studies (11, 29) on the basis of enterotoxin production. We screened for CFAs using antisera and determined the serotypes. We present here the associations encountered among toxins, CFAs, and serotypes.

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TABLE 1. Serotypes of 189 ETEC isolates with CFA/I and CS antigens

CFA and serotype	No. (toxin) of isolates from individuals from:	
	Egypt, 1989	Saudi Arabia, 1990
CFA/I		
O6:H16	1 (LT-ST)	1 (LT-ST)
O8:H9	1 (ST)	
O8:H17	1 (ST)	
O8:NM	1 (LT-ST)	
O126:HND	1 (ST)	
O126:NM	2 (ST)	
O128:H12		9 (LT-ST)
O153:H10		1 (ST)
O153:H45	1 (ST), 1 (LT-ST)	
O153:NM		2 (ST)
CFA/II		
CS1, CS3		
O6:H16	1 (LT), 3 (LT-ST)	3 (LT-ST)
O20:NM	1 (LT)	1 (LT-ST)
O81:H33	1 (LT-ST)	
O157:NM	1 (ST)	
O160:H10	1 (LT-ST)	
CS2, CS3		
O6:H16	4 (LT-ST)	29 (LT-ST), 1 (ST)
O8:NM		2 (ST)
O11:H33		1 (ST)
O18:NM		1 (LT-ST)
O140:H20	1 (ST)	
O151:H17	1 (LT-ST)	
O158:H10	1 (LT-ST)	
O163:H5	1 (ST), 1 (LT-ST)	
CS3		
O6:H16	1 (LT-ST)	3 (LT-ST)
O6:NM		1 (LT-ST)
O8:H9	2 (LT-ST)	1 (LT-ST)
CFA/IV		
CS4, CS6		
O8:H21	1 (ST)	
O8:NM		1 (ST)
O20:H10	1 (ST)	
O25:NM		1 (ST)
O27:H7		1 (ST)
O27:H20	1 (ST)	1 (ST)
O148:H28	1 (ST)	
O163:H34		1 (ST)
O?:H48		1 (ST)
CS6		
O6:H16	1 (ST)	1 (LT-ST)
O8:H32		1 (ST)
O8:NM	1 (LT-ST)	7 (ST)
O11:H33		1 (LT)
O20:NM	1 (ST), 1 (LT-ST)	
O25:NM	1 (LT)	2 (LT)
O27:H7		3 (ST)
O27:H20	1 (ST)	1 (ST)
O115:H1	1 (ST)	
O115:H35		1 (ST)
O115:H40		1 (ST)
O128:NM		1 (ST)
O148:H28	1 (ST)	9 (ST)
CS6		
O148:NM		2 (ST)
O151:H17	1 (ST)	
O158:NM		1 (LT)
O159:NM		4 (ST)
O163:NM		1 (ST)
O166:H21	1 (LT-ST)	
O166:H33		1 (LT)
O167:HNT	1 (LT)	
No CFA		
O2:HNT	1 (ST)	
O6:H16		3 (LT), 1 (ST), 1 (LT-ST)
O8:H9	1 (LT-ST)	
O8:H21	1 (LT)	
O8:H32		3 (LT), 1 (LT-ST)
O8:H33		1 (ST)
O8:H40	1 (LT)	
O8:H?		1 (LT)
O8:NM	2 (LT-ST)	5 (ST), 1 (LT-ST)
O15:H21	1 (LT)	
O25:NM		2 (LT)
O73:H17	1 (LT)	
O81:H45	1 (LT-ST)	
O114:H21		1 (LT)
O128:H12		1 (LT), 3 (LT-ST)
O133:H2	1 (LT)	
O133:H33		1 (LT-ST)
O146:NM		1 (LT)
O148:H28		1 (LT-ST)
O149:NM		1 (LT)
O153:NM		1 (ST)
O158:H45	1 (LT)	
O159:NM		1 (LT)
O162:H27		1 (ST)
O162:NM	1 (LT)	
O rough:H27		1 (LT-ST)
O rough:NM	1 (LT)	

MATERIALS AND METHODS

Bacterial strains. The ETEC strains characterized in the present study were obtained from two prior investigations conducted in the Middle East. In those studies, diarrheal stool samples were obtained from two separate locations: (i) from a clinic which provided care for U.S. troops participating in the Operation Bright Star field training exercises 30 km west of Cairo, Egypt, in November 1989 (29) and (ii) from 12 health care sites located in diverse geographic regions of northeastern Saudi Arabia during Operation Desert Shield, September to December 1990 (11).

The ETEC isolates used as controls in the CFA assay were H10407 for CFA/I (6), M424C1 for CS1 and CS3 (16), C91f for CS2 (25), E8775 for CS4 and CS6 (32), and E17018A (20) and E11881C (28, 30) for CS6.

CFA expression. Bacteria were inoculated from Dorset egg yolk slants or glycerol stocks made from the slants and were stored at −70°C. They were grown briefly in LB and then replica plated onto CFA agar plates. After incubation at 37°C overnight, colonies were transferred to nitrocellulose membranes and were treated as described previously (32). The primary antisera recognized CFA/I, CS1, CS2, CS3, CS4, and CS6. These antisera were raised in rabbits inoculated

with purified CFAs or with bacteria expressing CFAs. Antibodies to antigens other than CFAs were removed by incubation with homologous bacteria lacking CFAs. Antisera were shown to be specific for CFAs by Western blot (immunoblot) reactions. Isolates expressing CS3 alone or in conjunction with CS1 or CS2 were considered to be CFA/II. Isolates expressing CS6 alone or with CS4 were considered to be CFA/IV.

Serotyping. O:H serotyping was performed at the National Institute of Health in Tokyo and the *E. coli* Reference Center at Pennsylvania State University.

RESULTS

In most cases, each stool sample generated colonies with identical or very similar toxins, CFAs, and serotypes. These colonies were then judged to represent one isolate. Fifty-seven ETEC isolates from 57 patients in Egypt in 1989 and 132 ETEC isolates from 124 patients in Saudi Arabia in 1990 were examined. The phenotypes of these isolates are given in Table 1.

Toxins. The distributions of toxins produced from ETEC isolated in Egypt and Saudi Arabia were similar and are given in Table 2. Both LT and ST toxins were produced in 44% of the isolates, ST alone was produced in 39% of the isolates, and LT alone was produced in 17% of the isolates.

CFAs. The distributions of CFAs were similar in Egypt and Saudi Arabia. In ETEC isolates from both countries (Table 2), CFA/I was least common, one or more of the CFA/II family of antigens was expressed by one-third of the isolates, the CFA/IV family was expressed in another one-third of the isolates, and almost a quarter of the isolates expressed none of the CFAs detectable by our assays. Individuals in Egypt yielded a larger proportion of ETEC isolates bearing CFA/I than did individuals in Saudi Arabia, and the ETEC isolates were scattered over a wider variety of serotypes. A slightly higher proportion of CFA/IV was found in individuals in Saudi Arabia than in individuals in Egypt.

The distributions of CS1 and CS2 in CFA/II isolates and CS4 in CFA/IV isolates are given in Table 2. CS3 was rarely found alone in CFA/II isolates; more than 85% of the CFA/II isolates expressed CS1 or CS2 along with CS3. Seventy to 85% of the CFA/IV isolates expressed CS6 alone; however, these may have CS5 or even unidentified surface antigens that have been observed on ETEC isolates that express CS6 (13).

Serotypes. The isolates from Egypt comprised 30 O:H serotypes, while isolates from Saudi Arabia included 32 O:H serotypes, resulting in a total of 57 distinct O:H serotypes from the two locations. There were 32 O serogroups. O6:H16 was the predominant serotype from both Egypt (19% of the total) and Saudi Arabia (32%).

Association of toxin, serotype, and CFAs. The distributions of CFAs from the two sites for each toxin type (LT-ST, ST, or LT) are shown in Fig. 1. Expression of LT-ST was associated with CFA/II, and expression of ST was associated with CFA/IV. Isolates that expressed LT only were likely to lack CFA/I, CFA/II, or CFA/IV.

O6:H16 LT-ST CFA/II was the most frequent isolate from individuals in both Egypt and Saudi Arabia: O6:H16 LT-ST CFA/II accounted for 14% of the ETEC isolates from Egypt and 27% of the ETEC isolates from Saudi Arabia. These ETEC isolates expressed either CS1 and CS3, CS2 and CS3, or CS3 alone. CFA/IV was frequently encountered among isolates and was strongly associated with ST expression. There was no one frequent CFA/IV phenotype; CFA/IV was spread among several serotypes: O8:NM, O27:H7, O148:H28, and O159:H− as well as seven other serotypes (Table 1). CFA/I occurred least frequently, but most of the CFA/I isolates from individuals in Saudi Arabia were O128:H12 and LT-ST, making O128:H12 CFA/I LT-ST the third most common phenotype from Saudi Arabia. ETEC isolates from Egypt bearing CFA/I were as likely to express ST alone as LT-ST and were of a variety of serotypes.

TABLE 2. Toxin, CFA, and serotype distributions in 189 ETEC isolates

Toxin, antigen, or serotype	No. (%) of isolates from individuals from:		
	Egypt, 1989	Saudi Arabia, 1990	Combined
Toxin			
LT-ST	25	59	84 (44)
ST	20	53	73 (39)
LT	12	20	32 (17)
CFA			
CFA/I	9	13	22 (11)
CFA/II	20	44	64 (34)
CS1, CS3	8	4	12
CS2, CS3	9	35	44
CS3	3	5	8
CVA/IV	15	43	58 (31)
CS4, CS6	4	6	10
CS6	11	37	48
No CFA	13	32	45 (24)
Serotype			
O6:H16	11	43	54 (29)
O8:NM	4	16	20 (11)
O128:H12		13	13 (7)
O148:H28	2	10	12 (6)
O25:NM	1	5	6 (3)
O8:H9	4	1	5 (3)
O8:H32		5	5 (3)
O159:NM		5	5 (3)
O20:NM	3	1	4 (2)
O27:H7		4	4 (2)
O27:H20	2	2	4 (2)
O153:NM		3	3 (2)
O8:H21	2		2 (1)
O11:H33		2	2 (1)
O126:NM	2		2 (1)
O148:NM		2	2 (1)
O163:H5	2		2 (1)

Antibiotic susceptibility. Summaries of the antibiotic susceptibilities of the ETEC isolates examined in the present study have been reported elsewhere (11, 29). Except for O128:H12 and O8:NM isolates, there was no strong association between antibiotic susceptibility patterns and serotype, toxin, or CFA expression. All 11 of the O128:H12 CFA/I (or CFA$^-$) LT-ST isolates were resistant to all antibiotics tested except quinoline agents. All seven of the O8:NM CFA/IV (CS6) ST isolates were susceptible to all antibiotics tested.

Geographic distribution of ETEC isolates from Saudi Arabia. Unlike the ETEC isolates from 1989 that were from a single group of soldiers in Egypt, the ETEC isolates from

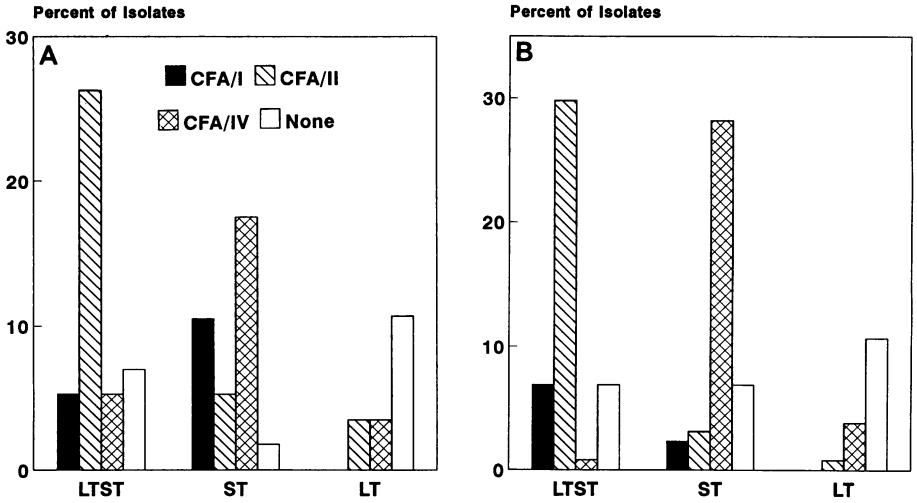

FIG. 1. Distributions of CFAs in ETEC isolates from Egypt (1989) (A) and Saudi Arabia (1990) (B) for each toxin type (LT-ST, ST, or LT).

1990 were from a large deployment of troops, with samples collected from multiple locations over a period of 4 months. Because of the wide diversity of samples, the data for Saudi Arabia were evaluated for clusters of similar ETEC strains by location, branch of military service, and month of collection. In general, no common toxin, serotype, or CFA predominated in any of the 12 collection sites, in any branch of the military, or during any of the four monthly periods. However, there was a cluster of 11 cases of multidrug-resistant O128:H12 CFA/I LT-ST from one location in Saudi Arabia during November.

DISCUSSION

The distributions of CFA/II, CFA/IV, and ETEC isolates lacking any of these CFAs were similar in Egypt and Saudi Arabia. However, there was a difference in the CFA/I distribution between the two sample populations. Individuals in Egypt yielded a larger proportion of ETEC isolates bearing CFA/I which were scattered over a wider variety of serotypes.

There were 98 distinct phenotypes among the 189 isolates. The greatest variety was in serogroups and serotypes; there were 32 distinct O serogroups and 57 distinct O:H serotypes. Within one serotype, there were generally multiple patterns of toxin and CFA expression, although one pattern sometimes predominated. These observations attest to the diversity of ETEC isolates in the Middle East, although some similarities between the Egyptian and Saudi Arabian samples may have been due to the fact that Egypt was one of the countries where food was obtained to feed the troops involved in Operation Desert Shield.

Most of the serotypes identified in the present investigation have been reported in other surveys, but O151:H17 and O11:H33 are new ETEC serotypes. Serotype O151:H17 was found twice in Egypt, once as CFA/II (CS2, CS3) LT-ST and once as CFA/IV (CS6) ST. O11:H33 was found twice in Saudi Arabia, once as CFA/IV (CS6) LT and once as CFA/II (CS2, CS3) ST.

An investigation of the distributions of CFA antigens in central Africa (17) yielded comparable proportions of CFA/I and CFA/IV but no CFA/II; however, PCFO159, PCFO166, and CS17 were found. We did not test for these antigens. A search of our data for isolates lacking CFAs to find serotypes commonly associated with the additional CFAs surveyed by McConnell et al. (17) was not fruitful. Only one isolate, O114:H21 LT, matched the serotype and toxin phenotypes and lacked CFAs; O114:H21 LT was likely to express CS17. Two isolates matched the phenotype for the CFA/III that occurs with CS6. A few isolates matched either the toxin or the serogroups associated with PCFO159 and PCFO166, but the H types or toxins did not match what has been reported previously. None of these represented a major proportion of the isolates.

From the present analysis, it seems that the isolates which lacked CFA/I, CFA/II, and CFA/IV cannot be accounted for by these additional CFAs. However, many of the ETEC isolates lacking CFAs had serotypes and toxin expressions that matched those of other strains in the collection that did express CFA/I, CFA/II, or CFA/IV. Since CFA expression is readily lost, it seems likely that some of these strains originally had one of the CFAs.

The phenotypes that were common in the present investigation have generally been reported in studies from other locations. *E. coli* O6:H16 CFA/II LT-ST has been reported in Thailand (2, 19), Myanmar (Burma) (17), India (24), Bangladesh (19), Argentina (1), and Peru (17). O128:H12 has been seen associated with CFA/I and LT-ST from Thailand (2, 19) and with CFA/I in Asia (31), Myanmar (17), and Bangladesh (19). Notable exceptions are O8:NM CFA/IV ST. *E. coli* O8 has not previously been reported with CFA/IV. The other serotypes found to be associated with CFA/IV have been reported by others: O27:H7 CFA/IV ST from Argentina (1) and from Zimbabwe (Rhodesia) and Zaire (17) and O148:H28 CFA/IV ST in Bangladesh (18) and Argentina (1). O148:H28 was one of the first ETEC isolates described, initially in Aden from British soldiers (22) and later from U.S. troops in Vietnam (4).

There is evidence that CFAs, LT toxoid, and O:H antigens are protective antigens (3, 7, 14, 21), and oral vaccines that will carry these antigens are being developed. The data presented here indicate that for vaccines to be effective in the Middle East, a wide variety of antigens must be included. While serotypes such as O6:H16 should be included in a vaccine, other antigens with broader distributions, such as

the toxins and CFAs, must be included. A vaccine composed of CFA/I, CFA/II components CS1, CS2, and CS3, and CFA/IV components CS4 and CS6 would have protected against 75% of the ETEC isolates encountered in the Middle East. A vaccine that also carried the LT B subunit would have protected against 90 to 95% of the ETEC isolates. Only the isolates expressing ST alone and none of the CFAs would be refractory to such a vaccine.

Although the ETEC isolates from the two sites obtained 1 year apart were similar, it does not follow that these data are totally predictive of ETEC isolates in the Middle East. Stoll et al. (26) showed that phenotypes of ETEC vary from season to season and year to year. Therefore, a vaccine designed for use in the Middle East could require antigens in addition to the ones identified in the investigations of the two areas.

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